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Presentation of a horse cytochrome *c* peptide by multiple H-2^b class I major histocompatibility complex (MHC) molecules to C57BL/6- and bm1-derived cytotoxic T lymphocytes: presence of a single MHC anchor residue may confer efficient peptide-specific CTL recognition*

In this study the immunogenic tryptic fragment from a horse cytochrome *c* (cyt *c*) digest recognized by cytotoxic T lymphocytes (CTL), induced by *in vitro* peptide stimulation from C57BL/6 (B6) and mutant B6.C-H-2^{bm1} (bm1) mice is identified. An identical sequence, p40–53, is recognized by CTL from both B6 and bm1 mice. In addition, both B6 and bm1 cloned CTL lines display unusual major histocompatibility complex (MHC) class I-restricted recognition of this peptide in that they respond to it in the context of H-2K^b, H-2D^b, and H-2K^{bm1} class I molecules, although the sequence lacks the usual structural K^b and D^b peptide-binding motifs. Truncated analogues which resemble the lengths of naturally processed MHC class I-presented peptides, confer reactivity for B6 and bm1 CTL against EL4 (H-2^b) targets as well as the L cell transfectants, L+K^b, L+D^b, and L+K^{bm1}. The antigenic peptide with the greatest potency is p41–49, which appears to be generated by angiotensin converting enzyme cleavage of the full-length p40–53 tryptic peptide. The minimum antigenic peptide recognized by both B6 and bm1 CTL, and which targets lysis on each of the transfectants, is the hexamer p43–48 peptide from horse cyt *c*. Residues Pro^{44} and Thr^{47}, which occupy polymorphic positions with respect to other species-variant cyt *c* molecules, influence recognition of these peptides differently for the B6 and bm1 CTL. The ability of H-2K^b, H-2D^b, and mutant H-2K^{bm1} class I molecules to present the same peptide to a single cloned CTL is discussed in the context of current knowledge of peptide anchor residues and side chain-specific binding pockets in the MHC class I peptide-binding site.

1 Introduction

Since Townsend and coworkers [1, 2] first demonstrated that CTL respond to peptide antigens, a large effort has been devoted to the task of determining which structural features of peptides allow them to be selected for presentation to CTL by MHC class I molecules. Several studies have established specific criteria for identifying peptides which might bind with high affinity to the peptide-binding cleft of an MHC class I molecule (reviewed in [3]). An important outcome of these studies is the identification of preferred structural motifs for natural peptides, comprised of two or more dominant "anchor" residues, which contribute to allelic MHC class I specificity in peptide presentation. Furthermore, natural peptides isolated from the peptide-binding cleft of MHC class I molecules are of limited length, generally from eight to ten residues [4].

X-ray crystallographic analyses of the structure for several human HLA class I molecules by Wiley and coworkers [5–7] have identified six peptide-binding pockets (designated A–F) within the antigen-binding cleft. In general, peptides appear to be "tethered" within the cleft by interactions between the amino end of the peptide to pocket A and by binding of the carboxyl end to pocket F of this cleft, allowing the central residues to bulge out of the cleft where they are exposed to solvent or TCR [7]. Together, these studies indicate that such peptide-binding pockets are critical determinants for allele-specific peptide binding within the MHC class I cleft, thereby strongly influencing the manner in which a peptide is presented to a responding MHC class I-restricted CTL. More recently, the X-ray crystal structures for several MHC class I/peptide complexes with a homogeneous peptide bound in the cleft [7–11] have provided a clearer understanding of how the peptide backbone and side chains interact with MHC class I residues within the antigen-binding cleft. Differences in the size and depth of the peptide-binding pockets allow for diversity in the peptide residues which can be accommodated by various MHC class I molecules, perhaps influencing the binding configurations of peptides within the MHC class I cleft. Other studies suggest that certain conformations of peptide/MHC class I complexes may be preferentially recognized by different CTL [12, 13].

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In the present study we have used *in vitro* peptide stimulation with a horse cytochrome *c* (cyt *c*) tryptic digest to examine the $CD8^+$ CTL response to peptides from horse cyt *c* presented by H-2K^b, H-2D^b, and mutant H-2K^{bm1} class I molecules. Our analysis of cyt *c*-specific CTL clones obtained from both B6 and bm1 anti-horse cyt *c* cultures reveals the unusual characteristic that a single tryptic fragment, p40–53, is recognized in the context of K^b, D^b, and K^{bm1} class I molecules by the same CTL although it lacks the complete structural K^b and D^b peptide binding motifs postulated by Rammensee and co-workers [4]. Studies with inhibitors of the serum-derived angiotensin-converting enzyme (ACE), captopril and enalapril, indicate that the product truncated by ACE, peptide p40–49, is probably the target peptide in the tryptic digest of horse cyt *c*. Using synthetic peptides we have shown that shorter analogues of p40–49 can also target cells expressing these alleles for lysis, although the p41–49 nonamer shows the strongest reactivity for CTL responses on all three class I molecules. It should be noted that there are no complete representations of the consensus motifs previously identified for K^b- and D^b-bound natural peptides among any tryptic peptide fragments from horse cyt *c*. Thus, the absence of such peptides, which could compete for occupancy of the MHC class I molecules, may allow the immunogenicity of other peptides to emerge. As a general phenomenon, the emergence of such cryptic peptides as immunogens could have significant implications for peptide vaccine design.

2 Materials and methods

2.1 Animals

Most mice used in this study were obtained from the breeding colony maintained at West Virginia University Health Sciences Center Vivarium. Some mice of the mutant strain, B6.C-H-2^{bm1}, were generously provided by Dr. Anna Seitz, University of Massachusetts, Amherst, MA. and Dr. Stanley G. Nathenson, Albert Einstein College of Medicine, Bronx, NY.

2.2 Cell lines

The following tumor cell lines (which express class I but not class II H-2 molecules) were maintained in suspension: EL4 (H-2^b), P815 (H-2^d), and CBA.D1 (H-2^k). Transfected cell lines were derived from a thymidine kinase-negative (TK^-) L cell fibroblast line, designated $LMTK^-$, and were grown as adherent cultures. These transfected cell lines, which include L+K^b, L+D^b, and L+K^{bm1}, were kindly provided by Dr. S. G. Nathenson.

2.3 Preparation of cyt *c* tryptic digests

A commercially available preparation of purified cyt *c* (Sigma) was resuspended at 10 mg/ml in 50 mM NH_4HCO_3. Trypsin was added to the cyt *c* solution to a final concentration of either 1% or 5% (w/w) trypsin. The pH was adjusted, as necessary, to pH 8.3–8.5 with 2 N NaOH, and the digestion was performed at 37°C with continuous mixing for 24 h. The tryptic digest was then lyophilized overnight and resuspended to 10 mg/ml in PBS.

2.4 Preparative HPLC analysis and isolation of fractions

The cyt *c* tryptic digest (10 mg) was applied to an octadecylsilane semi-preparative HPLC column (Vydac, The Sep/a/ra/tions Group; Hesperia, CA), and individual fractions were eluted at 2 ml/min with a 0–70% acetonitrile gradient over a time course of 90 min. Each fraction was lyophilized and resuspended to a molar concentration of 10 mg equivalents/ml in PBS.

2.5 Synthesis of peptides

Peptides were synthesized as previously described [14, 15] using a standard manual solid-phase synthesis procedure on a polystyrene-co-1% divinylbenzene resin and tert-butyloxycarbonyl for all N^α-protection of amino acids. Couplings were carried out using N-N″-diisopropylcarbodiimide and were monitored by ninhydrin reaction [16]. Simultaneous resin cleavage and side-chain deprotection was achieved by the high-low hydrogen fluoride method [17]. The crude products were purified to 98% purity by reverse-phase HPLC as previously described [14, 15], and peptide compositions were verified by amino acid analysis.

2.6 *In vitro* peptide stimulation

Primary (1°) cultures of spleen cells stimulated with a tryptic digest of horse cyt *c* were established for the induction of cyt *c*-specific CTL by *in vitro* peptide stimulation. Briefly, $8 \times 10^7 - 10 \times 10^7$ spleen cells were added together with 4 μM cyt *c* tryptic digest in a volume of 10 ml RP-10 per T-25 flask. Secondary (2°) cultures were established by the addition of 5×10^6 1° effectors and 5×10^7 irradiated (3000 rad) syngeneic spleen cells per flask in 10 ml RP-10 plus 4 μM antigen. All subsequent cultures were established in 24-well plates by the addition of 4×10^5 effectors plus 5×10^6 irradiated syngeneic spleen cells per well with 4 μM horse cyt *c* tryptic digest in RP-10 media, supplemented with 5% rat Con A supernatant and 50 mM α-methyl mannoside.

2.7 Isolation and maintenance of cloned CTL

Peptide-specific CTL clones were obtained from established lines of both B6- and bm1-derived CTL effectors by limiting dilution culture, followed by subcloning. Briefly, limiting dilution cultures were established by stimulation with horse cyt *c* tryptic digest of 300, 100, 30, 10, 3 or 1 cell per well of CTL effectors added to 96-well flat-bottom plates (CoStar Data Packaging, Cambridge, MA). Each well contained, in addition to CTL effectors, 1×10^6 irradiated (3000 rad) B6 or bm1 spleen cells plus 2 μM horse cyt *c* tryptic digest plus 5% Con A SN and 50 mM α-methyl mannoside. Putative clones were selected from wells in which fewer than one-third of the wells showed positive growth, and each clone was subsequently subcloned in the same manner.

2.8 Analysis of lytic activity by ^{51}Cr-release assay

The lytic activity of CTL cultures and clones was assayed in a 3- or 4-h ^{51}Cr-release assay. Briefly, this is performed as follows: target cells are incubated in 100 µl volume RP-10 media plus 200 µCi ^{51}Cr/10^6 cells for 60 min at 37 °C, washed in PBS, and resuspended in 10 mls RP-10 media, followed by an additional 30-min incubation at 37 °C. Following a final wash in PBS, target cells are resuspended to 2×10^5 cells/ml in RP-10 media, and 50 µl/well is added to 96-well round-bottom microtiter plates. Cyt c digests and peptides to be tested as potential antigens are resuspended to an initial concentration of 8–16 µM in RPMI 1640 media plus 2 % bovine serum albumin without serum, and a 50-µl volume is added to each well containing ^{51}Cr-labeled target cells and incubated 20 min at 37 °C. Effector CTL populations and clones are resuspended in RP-10 to yield the final desired effector : target ratios, and 100 µl is added to each well as appropriate. Since the final volume in each well is 200 µl, the final antigen concentrations tested in this assay are 2–4 µM. After incubation at 37 °C in 7 % CO_2 for 3–4 h the assay plates are centrifuged for 7 min. One-half the volume (100 µl) is collected from each well and transferred to 6 × 50 mm tubes for determination of total ^{51}Cr counts on an LKB Clinigamma Model 1272 counter (LKB Instruments, Finland). Background radioactivity is determined by collecting supernatants from wells in which RP-10 media was used in place of effector CTL. Maximum counts are determined from wells to which 100 µl of Triton X-100 was added in place of effector CTL. The determination of specific lysis for these samples is calculated according to the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{background release}}{\text{maximum release} - \text{background release}} \times 100$$

3 Results

3.1 B6- and bm1-derived CTL differ in their responses to a panel of species variant cyt c tryptic digests

Peptide-specific CTL from female mice of the C57BL/6 and bm1 mutant strains were induced by primary *in vitro* stimulation of spleen cells with a 4 µM concentration of horse cyt c tryptic digest and maintained by subsequent weekly restimulation with a 2 µM concentration of the same digest. To localize the region of horse cyt c which contains the antigenic tryptic fragment(s), both CTL effectors were tested initially for their lytic activity against EL4 targets in the presence of cyt c tryptic digests (2 µM) from cow, chicken, horse and pigeon. Peptide-specific CTL from B6 mice respond equally well to tryptic digests of cyt c from horse and cow but are unreactive against pigeon and chicken cyt c tryptic digests. The bm1-derived CTL response, however, is confined solely to the horse cyt c digest (Fig. 1). The response of both effectors against a tryptic digest of the p1-65 CNBR fragment of horse cyt c, but not p66–80 or p81–104 (data not shown), suggested that the tryptic fragment p40–53 is the most likely target antigen for both B6-derived and bm1-derived peptide-specific CTL because it contains sequence differences between all four of these species (Fig. 2) which are consistent with the reactivity pattern of the CTL.

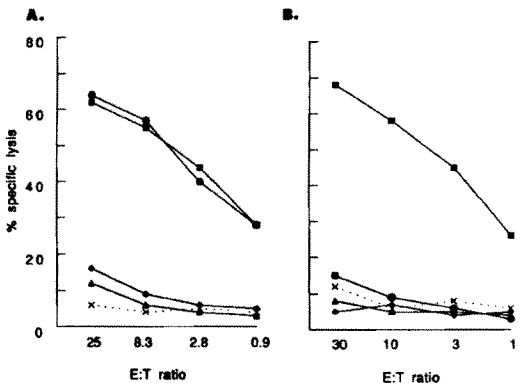

Figure 1. Response of (A) B6-derived and (B) bm1-derived CTL against EL4 target cells in the presence of cyt c complete tryptic digests from horse (■----■), bovine (●----●), chicken (▲----▲), and pigeon (◆----◆) species in a 3-h ^{51}Cr-release assay; (x----x) incicates their response to RP-10 media without antigen.

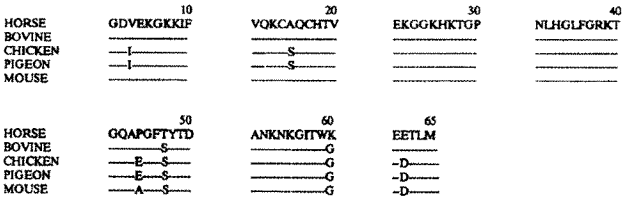

Figure 2. Comparison of primary sequences from horse, bovine, chicken, pigeon, and mouse species of the CNBr fragment, p1–65, from cyt c.

3.2 The same tryptic peptide is immunogenic for both B6- and bm1-derived CTL clones

Cloned CTL from both B6-derived and bm1-derived cultures were obtained by limiting dilution culture and maintained by weekly stimulation in the presence of 2 µM horse cyt c tryptic digest with 5 % Con A SN and 50 mM α-methyl mannoside, as described in Sect. 2.7. Several CTL clones exhibiting the same reactivity as the bulk CTL effectors were derived from each CTL population. Two clones, designated B6.H-4.1c and bm1.H-10.5c, were selected as representative cloned CTL effectors for further analysis.

To identify the precise peptide recognized by these cloned CTL lines, we assessed their reactivity on ^{51}Cr-labeled EL4 targets in the presence of individual fractions obtained from a preparative HPLC analysis of the horse cyt c digest (Fig. 3). The major peaks in the HPLC profile, fraction nos. 17, 18, 20, 22, 25 and 28 (Fig. 3A), were tested for their reactivity at a molar equivalent of 2 µM on ^{51}Cr-labeled EL4 targets. As shown in Fig. 3B and C, only fraction 20 is reactive with both B6-derived and bm1-derived CTL against EL4 targets, suggesting that the same peptide may be recognized by clone B6.H-4.1c and clone bm1.H-10.5c.

Amino acid composition analysis and sequence comparison performed on this fraction indicated that it contains a single peptide, the p40–53 tryptic fragment, which has the following sequence: Thr-Gly-Gln-Ala-Pro-Gly-Phe-Thr-

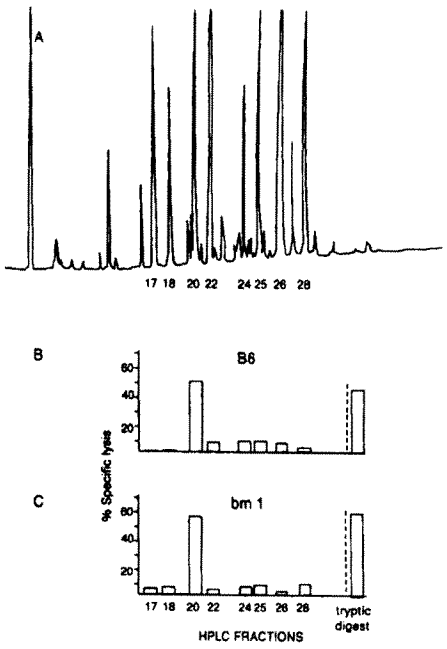

Figure 3. Analysis of HPLC-derived fractions from a complete tryptic digest of horse cyt *c* for their ability to target CTL lysis of EL4 targets in a 3-h ^{51}Cr-release assay. (A) HPLC profile of horse cyt *c* tryptic digest showing eight major peaks isolated by preparative HPLC. (B) Response of B6-derived CTL directed against the eight HPLC-derived fractions and the complete tryptic digest of horse cyt *c*. (C) Response of bm1-derived CTL against eight HPLC fractions and the horse cyt *c* complete tryptic digest.

Tyr-Thr-Asp-Ala-Asn-Lys. To determine why B6 CTL can respond to this peptide from both horse and cow, but bm1 CTL respond only to this peptide from horse cyt *c* (and neither responds to chicken or pigeon cyt *c* digests), we examined the primary sequence of the tryptic fragment p40–53 in each cyt *c* species. As shown in Fig. 2, there are two polymorphic residues at positions 44 and 47, both of which are contained within the antigenic p41–49 peptide. The immunogenic peptide from horse cyt *c* contains the residues Pro^{44} and Thr^{47}, whereas cow cyt *c* has Pro^{44} and Ser^{47}. Both chicken and pigeon cyt *c* contain Glu^{44} and Ser^{47}. This indicates that a proline at position 44 may be required for recognition of the horse cyt *c* peptide 40–53 by the B6-derived CTL, but that either threonine or serine at position 47 can be tolerated. Recognition of p41–49 by the bm1-derived CTL, however, is abrogated by the single $Thr^{47} \rightarrow$ Ser change between horse and cow cyt *c*.

3.3 Cloned B6- and bm1-derived CTL recognize horse cyt *c* p40–53-derived peptides presented by three H-2^b class I molecules

That these CTL are self-MHC class I restricted is established by their response in the presence of antigen to syngeneic EL4 (H-2^b), but not to allogeneic P815 (H-2^d) or CBA.D1 (H-2^k), targets (data not shown). In addition, no reactivity was observed on syngeneic EL4 targets in the presence of undigested, native horse cyt *c* (data not shown). We, thus, examined the reactivity of the B6.H-4.1c and bm1.H-10.5c CTL clones on L cell (H-2^k) targets transfected with either the H-$2K^b$, D^b, or K^{bm1} class I molecules to determine the H-2^b class I-restriction phenotype for these clones.

As shown in Figs. 4A and B, both CTL clones respond effectively to all three transfected targets in the presence of the horse cyt *c* peptide p41–49 suggesting that all three class I molecules can be used as restriction elements for their response to either the tryptic digest or peptide. Other B6-derived and bm1-derived CTL clones, as well as several subclones derived from B6.H-4.1c and bm1.H-10.5c (data not shown), also displayed a similar multiple restriction pattern on $L+K^b$, $L+D^b$, and $L+K^{bm1}$ targets in the presence of the horse cyt *c* tryptic digest and p41–49 peptide.

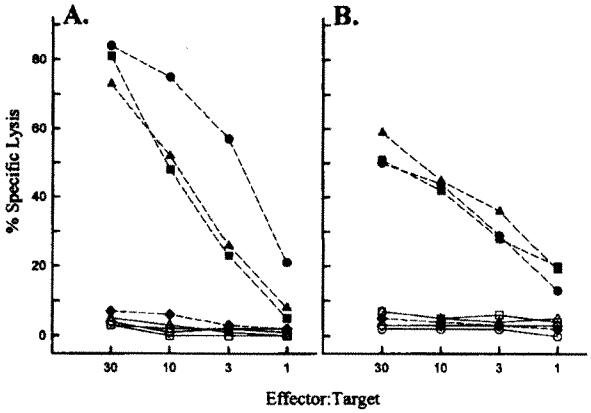

Figure 4. Response of (A) CTL clone B6.H-4.1c and (B) CTL clone bm1.H-10.5c in a 3-h ^{51}Cr-release assay against $L+K^b$ (circle symbols), $L+D^b$ (triangle symbols), $L+K^{bm1}$ (square symbols), and $LMTK^-$ (diamond symbols) targets, in the presence (closed symbols) or absence (open symbols) of 2 μM horse cyt *c* p41–49.

3.4 The hexamer p43–48 peptide from horse cyt *c* constitutes the minimum antigenic sequence recognized by both B6 and bm1 CTL

To determine the minimum peptide which targets lysis for both B6 and bm1 CTL, we tested both clones for their reactivity on a panel of truncated peptides derived from the horse cyt *c* p40–53 tryptic fragment. As shown in Table 1, the peptides p41–49 and p41–48, which resemble the naturally processed peptide length [18, 19], are fully reactive at 2 μM concentration in targeting the responses of both CTL clones on EL4 targets. Furthermore, the truncated peptides, p42–48 and p43–48, are also reactive, although diminished in their antigenicity for both CTL clones. Both p41–47 and p44–49 truncated peptides, however, are unreactive with respect to stimulating CTL-mediated lysis of these targets by both clones B6.H-4.1c and bm1.H-10.5c. Thus, these results allow us to define the amino and carboxyl termini of the minimum antigenic peptide sequence, for both B6- and bm1-derived anti-horse cyt *c* CTL, as residues Ala^{43} and Tyr^{48}, respectively. Furthermore, this p43–48 peptide serves as the minimal antigenic peptide sequence for all three transfected cell lines, $L+K^b$, $L+D^b$, and $L+K^{bm1}$, with lysis comparable to that shown on EL4 in Table 1 (data not shown).

Table 1. Reactivity of cloned CTL, B6.H-4.1c and bm1.H-10.5, to horse cyt *c* p40–53-derived peptides[a)]

Peptide	Clone B6.H-4.1c 3:1	1:1	Clone bm1.H-10.5c 3:1	1:1
p40–53	54	22	50	33
p41–49	53	25	60	37
p41–48	50	20	40	24
p42–48	37	13	10	3
p43–48	37	17	12	8
p41–47	2	0	1	1
p44–49	3	2	2	1

a) All peptides were tested at a final concentration of 2 μM, in a 3-h ^{51}Cr-release assay with EL4 (H-2^b) targets.

3.5 Replacement of Pro^{44} in the horse cyt *c* p41–48 octamer interferes with peptide recognition

To confirm the apparent importance of the Pro^{44} residue in CTL responses to this peptide, two analogues were synthesized in which the Pro^{44} residue was substituted with either Gly^{44} or Ala^{44}; designated p41–48/Gly^{44} and p41–48/Ala^{44}, respectively. The results depicted in Fig. 5 reveal that recognition of the EL4 target by clone B6.H-4.1c is abrogated by a Pro^{44} → Gly or Pro^{44} → Ala substitution in the native peptide. Also, clone bm1.H-10.5c (Fig. 5B) responds in a similar manner. Similar responses were observed when both clones were tested on L+K^b, L+D^b, and L+K^{bm1} targets (data not shown).

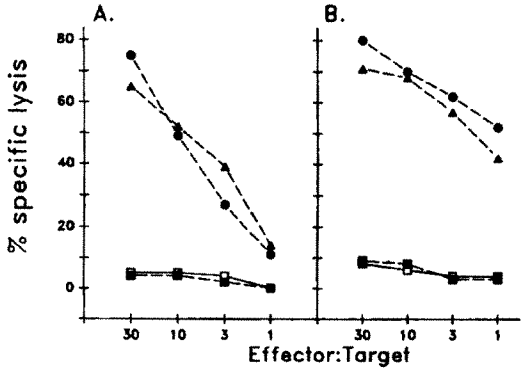

Figure 5. Response of horse cyt *c*-specific CTL clones B6.H-4.1c and bm1.H-10.5c on EL4 targets in the presence of 2 μM concentration of horse cyt *c* tryptic digest (●----●), horse cyt *c* p41–48 native peptide (▲----▲); and monosubstituted p41–48 peptide analogues: p41–48/Ala^{44} (□----□) and p41–48/Gly^{44} (■----■).

3.6 Residue Thr^{47} influences the length of the antigenic peptide recognized by B6-derived CTL

Since B6-derived cyt *c*-specific CTL respond to both horse and cow cyt *c* p40–53 tryptic peptides, we examined the reactivity of the p41–49 peptide from cow cyt *c* (which contains a Thr^{47} → Ser substitution) for its ability to target lysis by clone B6.H-4.1c against EL4 targets. The results shown in Table 2 demonstrate that this peptide is not recognized by clone B6.H-4.1c, although the related horse cyt *c* peptide is recognized. To determine why this single Thr^{47} → Ser substitution should influence the length of the minimum antigenic peptide, we examined several longer peptides derived from the cow cyt *c* sequence for their ability to target lysis of the EL4 target by clone B6.H-4.1c. Although the minimum antigenic peptide from cow cyt *c* was identified as p40–49 (Table 2), the addition of a carboxyl-terminal Asp residue in position 50 abrogates the ability of the resulting p40–50 peptide to effectively target lysis by clone B6.H-4.1c. This targeting activity is fully restored, however, with the addition of a carboxyl-terminal Ala residue in position 51.

Several recent studies [20–22] have characterized a serum-derived dipeptidase activity mediated by ACE, in generat-

Table 2. Reactivity of CTL clone B6.H-4.1c to cow cyt *c* p40–53-derived peptides[a)]

Peptide	Clone B6.H-4.1c 3:1	1:1
p40–53	52	31
p40–51	61	36
p40–50	10	3
p40–49	65	43
p40–48	1	0
p41–49	1	2

a) All peptides were tested at a final concentration of 2 μM, in a 3-h ^{51}Cr-release assay with EL4 (H-2^b) targets.

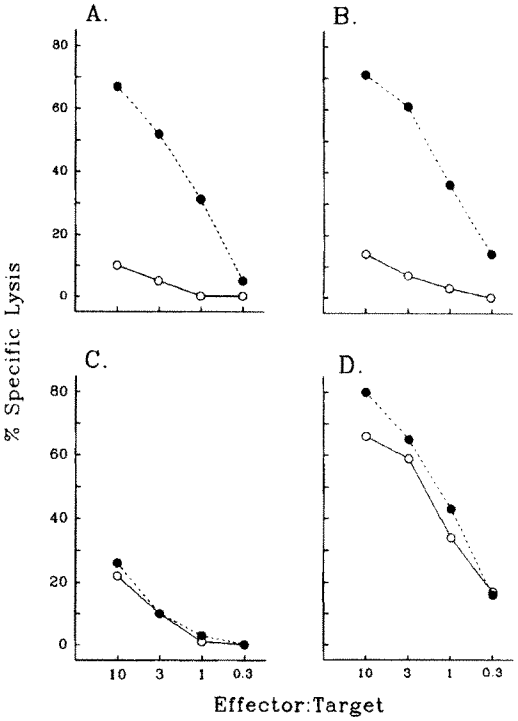

Figure 6. Effect of the addition of captopril on cow cyt *c*-derived peptide reactivity to clone B6.H-4.1c in a 4-h ^{51}Cr-release assay. Effector cells from clone B6.H-4.1c were tested against ^{51}Cr-labeled EL4 target cells with 2 μM peptide, as indicated, either in media alone (●----●) or in the presence of 100 μM captopril (○----○); (A) peptide p40–53 from cow cyt *c*; (B) peptide p40–51; (C) peptide p40–50; and (D) peptide p40–49.

ing antigenic fragments from longer peptides *in vitro*. Thus, the reactivity observed with p40–51 might result from cleavage of the two carboxyl-terminal residues through this dipeptidase activity to yield the optimal p40–49 peptide from cow cyt *c* as a target antigen for lysis by clone B6.H-4.1c. From the p40–50 peptide this ACE activity would yield the nonamer p40–48, which is not recognized by B6 CTL. To examine this possibility, we determined the effect of the ACE inhibitor, captopril, on peptide recognition with EL4 target cells by clone B6.H-4.1c. (Fig. 6). The activity of ACE is evident for both the p40–53 tryptic fragment in the cow cyt *c* tryptic digest and the truncated peptide p40–51, as indicated by inhibition of their reactivity with captopril (Fig. 6A and B). Furthermore, the activity of ACE apparently is responsible for generating p40–49 as the target peptide, since addition of captopril to this truncated peptide fails to inhibit its activity (Fig. 6D). The diminished reactivity of the p40–50 analogue can be attributed to cleavage of these peptides by ACE to the inactive p40–48 form, whose reactivity is not restored by the addition of captopril (Fig. 6C). These observations were confirmed (data not shown) using the more selective and more potent ACE inhibitor, enalapril [23, 24]. Similar results were also obtained with the corresponding p40–50 peptide from horse cyt *c* and its analogue (data not shown), thereby discounting a possible effect attributed to the Ser^{47} substitution in the cow cyt *c* peptide. Thus, the serum-derived ACE generates an optimal form of the peptide recognized by clone B6.H-4.1c. Furthermore, the lack of reactivity of p40–50 is probably due to generation of peptide p40–48, which is not an effective antigen (Table 2), by the dipeptidase activity of serum-derived ACE.

3.7 Titration of p40–53-derived peptides reveals significant differences in their reactivity

The nonamer p41–49 from horse cyt *c* corresponds to the optimal size peptide of most natural D^b-binding peptides, and the octamer p41–48 fits the optimal size of most K^b-bound natural peptides. Although these two peptides yield maximum CTL-mediated lysis when added at 2 μM concentration, for both B6.H-4.1c and bm1.H-10.5c CTL clones, neither peptide exhibits the complete structural motif of two defined anchor residues predicted for natural peptides which bind to H-2K^b or H-2D^b [4]. Both peptides have no predicted dominant anchor residues [4] and only a single predicted strong anchor residue for natural peptides which bind to K^b and D^b class I H-2 molecules, Phe^{46} in position 5 or 6, respectively. Although the peptide p41–48 does not express any of the previously identified dominant anchor residues the octamer p42–49 displays a Phe at position 5, which has been identified as a dominant anchor residue for K^b-bound octamers. We are presently examining the effectiveness of the p42–49 peptide for its ability to be recognized by the horse cyt *c*-specific CTL.

To determine whether the absence of dominant anchor residues influences their antigenic potency horse and cow cyt *c*-derived peptides, whose recognition was not influenced by the presence of captopril (Fig. 6), were titrated from 8 μM to 8 pM concentrations on L+K^b, L+D^b, and L+K^{bm1} targets in a 4-h ^{51}Cr-release assay. The response of CTL clone B6.H-4.1c to these peptides was comparable on all four targets, with no significant differences in lytic activity throughout the peptide titrations. As no preference for the use of a single class I molecule as a restriction element can be discerned, it appears that K^b, D^b, and K^{bm1} are equally effective at binding and presenting these peptides.

The results in Fig. 7 show for L+K^b, L+D^b, and L+K^{bm1} targets that, although p41–48 and p41–49 demonstrate maximum targeting activity at a concentration of 2 μM, the two peptides differ significantly in their reactivity when titrated as peptide antigens. The octamer p41–48 peptide is effective at targeting the lytic response of clone B6.H-4.1c at nanomolar concentrations. The p41–49 nonamer peptide, however, shows significant lysis on all three targets with as little as 8 pM (Fig. 7 A–C), which is at least as potent as the reactivity observed with most natural MHC class I-presented peptides [3]. Thus, it is likely that the nonamer peptide, p41–49, from horse cyt *c* is the optimal target antigen for clone B6.H-4.1c. Furthermore, the three MHC class I molecules, K^b, D^b, and K^{bm1}, are comparable in terms of peptide presentation.

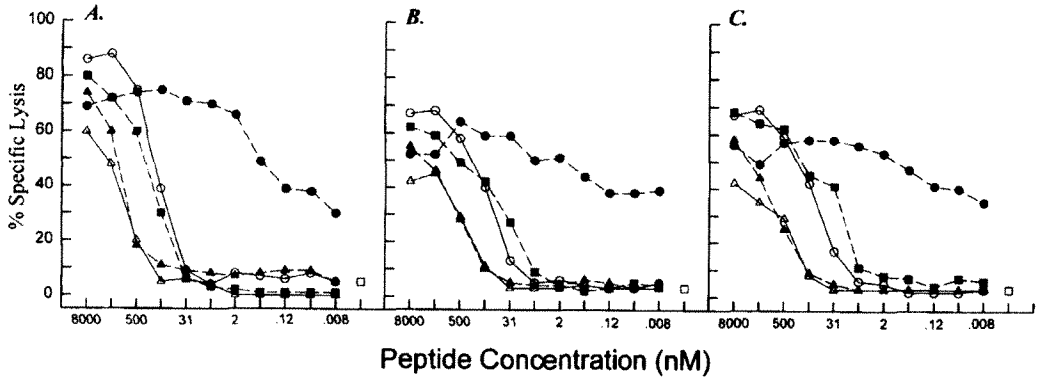

Figure 7. Peptide titration of clone B6.H-4.1c CTL response to horse and cow cyt *c* tryptic digests and p40–53-derived peptides. Clone B6.H-4.1c was tested at a 3:1 effector:target ratio in a 3-h ^{51}Cr-release assay on L+K^b (A), L+D^b (B), and L+K^{bm1} (C) target cells, in response to titrated concentrations of peptides from 8 μM – 8 pM. Horse cyt *c* p40–53-derived peptides: p41–49 (●------●); p41–48 (■------■); p42–48 (▲------▲); and p43–48 (△------△); cow cyt *c* peptide: p40–49 (○------○); and media alone (□).

With respect to the amount of peptide antigen which results in 30% lysis, the response with p41–48 (60 nM) requires approximately 7500-fold more peptide than that with p41–49 (8 pM) (Fig. 7 A–C). This observation further implicates residue Thr^{49}, in peptide position P9, as important for the CTL response to this peptide. The truncated peptides, p42–48 and p43–48, are far less active in this regard; each differing by approximately 100000-fold, respectively, from p41–49 (Fig. 7). A comparison of the cow cyt *c* p40–49 peptide tested in this manner (Fig. 7) reveals that p40–49, which differs from the native horse cyt *c* p41–49 sequence by a $Thr^{47} \rightarrow Ser$ substitution, as well as the addition of a Thr^{40} residue on the amino terminus, is 15000-fold less reactive than the horse cyt *c* peptide, p41–49.

4 Discussion

This study describes the response of CTL from B6 and mutant bm1 mice to the horse cyt *c* tryptic fragment, p40–53. We have shown that Pro^{44} and Thr^{47} residues, which differ among a wide range of species-variant cyt *c* molecules, play an important role in determining the reactivity of peptides derived from this tryptic fragment and that this peptide can be presented effectively to both B6 and bm1 CTL by either H-$2K^b$, H-$2D^b$, or H-$2K^{bm1}$ class I molecules expressed in the B6 and mutant bm1 strains. Thus, the response of cloned CTL from B6 mice to this peptide can be restricted by both self class I molecules, H-$2K^b$ and H-$2D^b$, and the mutant H-$2K^{bm1}$ molecule. Similarly, bm1 CTL are self-restricted to H-$2K^{bm1}$ and H-$2D^b$, as well as the wild-type H-$2K^b$ molecule. This unusual H-2 restriction phenotype differs, however, from the response of other "promiscuous" CTL [25–27] in that H-2^k and H-2^d class I allelic forms do not present this peptide to B6 or bm1 CTL.

The polymorphic residues, Pro^{44} and Thr^{47}, have different effects on peptide recognition by B6 and bm1 CTL. Although the same minimum antigenic sequence of the horse cyt *c* hexamer p43–48 can target lysis by CTL from both strains, an important difference between the two is discerned by their responses to various analogue forms of this peptide. Thus, a Pro residue in the polymorphic position 44 of both horse and cow cyt *c* is essential for B6 CTL recognition in that there is no response against tryptic digests of either avian cyt *c* ($Pro^{44} \rightarrow Glu$, see Fig. 2A), rabbit cyt *c* ($Pro^{44} \rightarrow Val$) or mouse cyt *c* ($Pro^{44} \rightarrow Ala$) (data not shown). For the bm1 CTL response, however, only the horse cyt *c* tryptic digest can target lysis on EL4 target cells (Fig. 2B). This indicates that a Thr residue in the polymorphic position 47 of horse cyt *c* (which is replaced by Ser^{47} in all other cyt *c* species tested) exerts a dominant influence for recognition by bm1 CTL. In addition, Pro^{44} also plays a role in the bm1 CTL response, since its replacement with Ala^{44} abrogates the recognition of the p41–48 peptide (Fig. 5). A secondary influence of Thr^{47} on B6 CTL recognition is suggested by the observation that its replacement with a Ser^{47} residue results in an increased length of the minimum antigenic peptide from p43–48 in horse cyt *c* to p40–49 in cow cyt *c* (Tables 1 and 2). Differences in the reactivity patterns for the B6- and bm1-derived CTL, as shown by the effects of Pro^{44} and Thr^{47} on CTL recognition of this peptide, suggest that they may respond differently to the same peptide/MHC class I conformation. Alternatively, perhaps they can recognize two distinct conformations of this peptide/MHC class I complex.

An important concern with respect to defining a consensus structural binding motif for MHC class I-bound peptides is the identification of residues whose positions are critical in anchoring the peptide within the cleft. For the binding of octamer peptides to H-$2K^b$, it is generally recognized that the dominant anchor residues are Phe or Tyr at position 5, and a Leu at position 8 [4]. For H-$2K^{bm1}$ binding, natural peptides lack the dominant anchor residues which characterize K^b-bound peptides, but they do express a single unique dominant anchor residue, Tyr, at position 7 [28]. Two residues, Asn at position 5 and Met at position 9, are the dominant anchor residues for D^b-bound nonamer peptides [14]. Thus, the various natural peptides isolated from these class I molecules have a strong preference for two anchor residues as part of a consensus motif, which appear to be lacking in the horse and cow cyt *c* peptides.

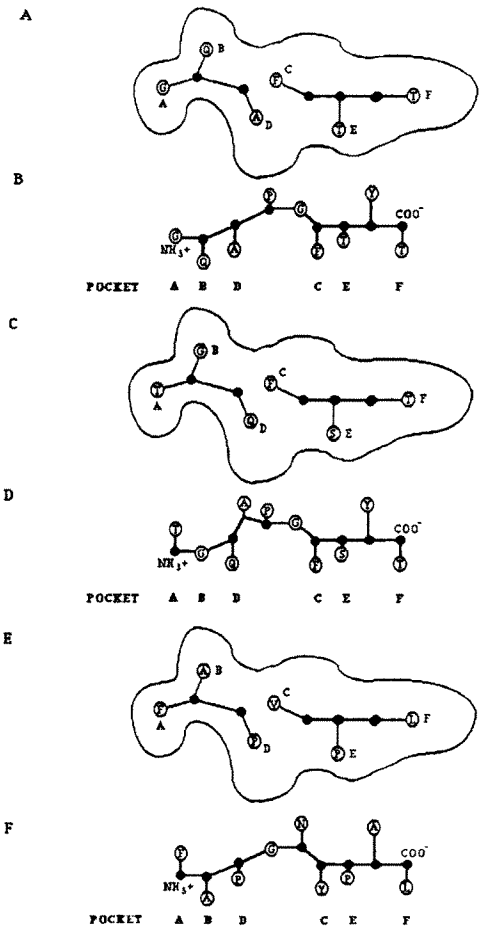

Figure 8. Peptides bound to the MHC class I H-$2K^b$ molecule and the possible positions of the residues (shown in the single letter code) with respect to binding pockets A through F; based on the interpretation of the crystal structure of SEV-9 bound to H-$2K^b$ by Matsumura et al. [20]. (A), (C) and (E) are viewed looking down on top of the molecules; and (B), (D) and (F) show side views of the peptide. Horse cyt *c* p41–49 is depicted in (A) and (B); cow cyt *c* p40–49 in (C) and (D); and, for comparative purposes, SEV-9 is shown in (E) and (F).

We have shown that the sequence with the greatest stimulatory activity for the cyt *c* peptide-specific B6- and bm1-derived CTL clones is the nonameric horse cyt *c* peptide p41–49. This peptide is one residue longer than most K^b-restricted peptides, but it does contain a bend-forming sequence (Pro^{44}-Gly^{45}) which has been postulated to facilitate the binding of longer peptide sequences to MHC class I molecules by allowing the extended peptide backbone to protrude out of the antigen-binding cleft [8, 29]. If this peptide is aligned within the K^b peptide-binding cleft in a similar orientation to the Sendai virus N peptide SEV-9 [8], Phe^{46} is in a position to occupy pocket C as a dominant anchor residue (see Fig. 8). Pocket F, however, would contain Thr^{49}, which lacks the hydrophobic character of most anchor residues found in this position. However, Thr^{47}, which we have shown to be crucial for recognition of this peptide in that cow cyt *c* p41–49 (containing Ser^{47}) is nonstimulatory, is well-positioned to interact with pocket E. This interaction may well compensate for the weaker peptide binding to pocket F.

An interesting consideration with respect to CTL recognition of this peptide is whether p41–49 might be naturally processed and presented if cyt *c* were introduced into the cytosolic MHC class I pathway. Preliminary studies indicate that when CTL induced with a tryptic digest of pigeon cyt *c* are tested for lysis of osmotically loaded EL4 cells, they demonstrate an effective lytic response (data not shown). We are presently examining whether CTL induced by such a naturally processed form of horse cyt *c* are also responsive to the p41–49 peptide identified as a dominant CTL-recognized antigen in this study.

It is intriguing that both SEV-9 and horse cyt *c* p41–49 bind to D^b as well as K^b class I molecules ([29] and this study), given that neither of these peptides contain the structural motifs predicted as necessary for binding to both K^b and D^b. Perhaps, however, the secondary structure conferred on these peptides by the β bend-forming "Pro-Gly" dipeptide combination allows them to fit into antigen-binding clefts of slightly different configurations. Deres et al. [29] suggested that the Pro residue in the SEV-9 peptide may have a critical role in peptide binding to the MHC class I cleft. Thus, although the orientation of p41–49 (shown in Fig. 8) places Pro^{44} in a position where it may directly interact with the TCR, the results of the studies based on the use of analogues substituted at position 44 with a Gly or Ala residue, may also be due to disruption of the bend-forming "Pro-Gly" dipeptide.

The accommodation of sequences containing the Pro-Gly sequence as longer peptides in the MHC class I cleft may also account for recognition of the decameric cow cyt *c* p40–49 peptide. Thus, although Ser^{47} (which has a smaller side chain than Thr^{47}) may weaken interactions with pocket E, this appears to be compensated by the addition of Thr^{40} which could displace Gly from position 1 in the peptide sequence. Presumably, the Gly-Gln-Ala interactions with pockets A, B, and D (see Fig. 8) are less favorable than Thr-Gly-Gln interactions at these sites.

The ability of these peptides to bind D^b molecules (given the absence of Asp, Leu or Met in this region of horse cyt *c*) is perhaps unexpected, but not without precedent. Although most self peptides isolated from $H\text{-}2^b$ class I MHC molecules conform to the dominant structural motifs, it has been demonstrated that alternative motifs may also exist [28]. A Phe residue (Phe^{46}) is found at position 6 in p41–49 and is also present as a strong anchor residue at position 6 in natural nonameric peptides found associated with D^b molecules [4]. Thus, this residue might also serve to anchor the p41–49 peptide to pocket C of the D^b molecule. That significant lysis can be observed in the presence of peptide concentrations in the picomolar range, despite the lack of a complete consensus motif characteristic of natural peptides bound to these MHC class I molecules, indicates that a peptide anchor residue in pocket C of the MHC class I cleft might be both necessary and sufficient for presentation of some peptides by certain MHC class I molecules for CTL recognition. Furthermore, our finding that all three class I molecules appear to be efficient restriction elements for this peptide suggests that pocket C interactions with peptide residues are important features for MHC class I-restricted recognition by CTL.

To our knowledge, the degenerate MHC restriction in the CTL response to peptide p41–49 constitutes the first demonstration that an $H\text{-}2K^b$-restricted CTL can respond to the same antigen in the context of the related $H\text{-}2K^{bm1}$ mutant class I molecule, as well as $H\text{-}2D^b$. In considering the possible molecular basis for this unusual degenerate MHC restriction, we noted several observations which suggest $H\text{-}2K^b$ and $H\text{-}2D^b$ class I molecules expressed on L cell transfected targets may be somewhat dissimilar in their peptide binding and/or TCR contact characteristics. Furthermore, whenever this difference occurs, the response on $L+K^{bm1}$ always resembles that directed against $L+D^b$ rather than $L+K^b$ targets. For example, lysis by clone B6.H-4.1c directed to the horse cyt *c* p41–49 peptide is approximately threefold greater against $L+K^b$ than either $L+D^b$ or $L+K^{bm1}$ targets (Fig. 4). This distinction between K^b and D^b, as well as the similarity between D^b and K^{bm1}, is consistently observed for this clone and for other B6-derived CTL clones with this and other peptides. These findings indicate that a structural similarity between $H\text{-}2D^b$ and $H\text{-}2K^{bm1}$ class I molecules, which is distinct from $H\text{-}2K^b$, may account for the unique observation that this peptide can be effectively presented by both K^b and K^{bm1} class I molecules to the same CTL.

Although the $H\text{-}2D^b$ primary sequence differs from $H\text{-}2K^b$ by approximately 20 %, the sequence differences between $H\text{-}2K^b$ and mutant $H\text{-}2K^{bm1}$ class I molecules are limited to three amino acid substitutions within a localized region of the long α-helix of the α2 domain [30]. The substitutions from $K^b \rightarrow K^{bm1}$ in these three positions are as follows: 152 (Glu → Ala), 155 (Arg → Tyr), and 156 (Leu → Tyr). An examination of these positions in the D^b primary sequence reveals that position 152 and 156 are occupied by the same residues found in K^{bm1}, and position 155 in $H\text{-}2D^b$ contains a His residue in place of the Tyr^{155} found in K^{bm1}. On the basis of X-ray crystallographic studies for $H\text{-}2K^b$/peptide complexes [8–10], it is clear that those residues in positions 152 and 156 are oriented in a manner such that their side chains are important in stabilizing peptide binding to pocket E within the antigen-binding cleft. The residue found in position 155, which differs between these two alleles, is oriented in such a way that it may be involved in TCR contact rather than peptide-binding [8–10]. Thus, it is likely that one or both residues situated in pocket E exert a

dominant efect on the presentation of the native horse cyt *c* peptide to B6- and bm1-derived CTL by H-2D^b and H-2K^{bm1} class I molecules. Consistent with the notion that interactions with pocket E are crucial to the binding of the cyt *c* peptides to these H-2^b molecules is the finding that the peptide with the greatest stimulatory activity, the nonameric sequence p41–49, has a Thr^{47} residue in the correct position for interaction with the E pocket (see Fig. 8). The same peptide from cow cyt *c*, which has a Ser^{47} with a smaller side chain, is not recognized by these CTL.

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